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2. Patent application number *(The Patent Office will fill in this part)* 9720693.2 30 SEP 1997

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

University of Wales College of Medicine
Heath Park
Cardiff
CF4 4XN

Patents ADP number *(if you know it)* -- 7985460003

If the applicant is a corporate body, give the country/state of its incorporation: United Kingdom

4. Title of the invention: Assay

5. Name of your agent *(if you have one)*: Wynne-Jones, Laine and James

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Morgan Arcade Chambers
33 St Mary Street
Cardiff
CF1 2AB

Patents ADP number *(if you know it)* 1792002

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
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Number of earlier application	Date of filing *(day / month / year)*
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

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Claim(s)	–
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11. I/We request the grant of a patent on the basis of this application.

Signature [signature] Date 30.9.97

Wynne-Jones, Lainé and James

12. Name and daytime telephone number of person to contact in the United Kingdom

W.J. Newell - Tel 01222 229526

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ASSAY

The present invention relates to an assay for measuring antibodies to the thyrotropin receptor and specifically to the use of a cell line transfected with α-luciferase cDNA therein.

The thyrotropin receptor (hereinafter 'TSH-R') is known to regulate both the function and proliferation of the thyroid cell, and is stimulated by the hormone thyrotropin (TSH). The TSH-R is also a target for autoantibodies that either block the action of TSH (TBAb) (ie. act as hypostimulants or inhibitors) or stimulate (hyperstimulate) the thyroid (TSAb).

Stimulation by TSAb is believed to be a mechanism operating in Graves' disease, whilst inhibition by TBAb is believed to be the case in idiopathic myxoedema. Patients with hyperthyroid Graves' disease produce antibodies which mimic the action of TSH, leading to chronic stimulation of adenylate cyclase; and whereas the autoantibodies in some patients with idiopathic myxoedema are also able to bind to the TSH-R, nevertheless this does not result in an increase in intracellular cyclic adenosine 3',5'-monophosphate (cAMP).

Many assays have been developed to measure TSH-R autoantibodies. The most widely used is a radioreceptor assay in which the binding of bovine ^{125}I-TSH to detergent-solubilised porcine TSH-R is inhibited by immunoglobulins or sera from patients suspected of having TSH-R autoantibodies. The main problems with this assay are that it uses a non-

human antigen; and that it measures binding and not biological activity, hence it is not able to distinguish between TSAb and TBAb.

It is clearly desirable to be able to distinguish between stimulation and inhibition, and therefore attempts have been made to develop a bioassay in which an effect of TSH (or its inhibition by TBAb) or TSAb is measured, such as the increase in cAMP. This may be done in thyroid slices or thyroid cells in culture and the greatest sensitivity, defined as the highest percentage of Graves' patients being positive, is achieved when the assay is performed in hypotonic medium. Both the radioreceptor and the bioassays are hampered by limited availability of biological material. To overcome this, a rat thyroid cell line (FRTL-5) has been developed, but here species differences may still be problematic. The recent cloning and sequencing of the TSH-R give unlimited access to recombinant human TSH-R.

As described by Libert *et al* in Biochem. & Phys. Res. Comm. 165 (3) 1250-1255 (1989) [all references herein are herein incorporated in their entirety, together with any cross-references therein], previous cloning of the dog thyrotropin receptor opened the way to molecular characterisation of the human TSH-R *via* isolation of human TSH-R cDNA clones; the analysis of the primary structure of the encoded polypeptide; and evidence that the recombinant molecule binds auto-antibodies found in patients with Graves' disease and idiopathic myxodoema. The dog TSH-R cDNA (a 2.8 kb fragment) was used to hybridise a human thyroid DNA library. Sequencing of the resulting clones gave rise to a 2292 nucleotide residue open reading

frame encoding a 744 amino acid polypeptide having 90.3% convergence with the dog TSH-R. Transfection of the coding sequence in the pSVL vector of COS-7 cells allowed confirmation of the protein's ability to bind specifically TSH.

The cotransfection of Chinese hamster ovary (CHO) cells with a pSVL vector containing the coding region of human TSH-R led to the selection of cell lines particularly responsive to TSH or TSAB in terms of their cAMP accumulation (reported by Perret *et al* in Biochem. & Biophys. Res. Comm. 171 (3) 1044-1050 (1990)). Dose response curves of TSH-mediated cAMP accumulation were reported for clones JP14, JP26 and JP28, and the number of receptors per cell were found to be highest in clones JP14 and JP09.

This work gave rise to the possibility of a bicassay in which cAMP production is measured in cells stably transfected with the human TSH-R in the presence of an autoantibody either alone (TSAB) or in the presence of TSH (TBAB), (Ludgate *et al* in Molec. & Cell. Endocrin. 73 R13-R18). However, such an assay is not sufficiently robust for routine use since it takes several days to perform and requires tissue culture facilities.

Accordingly, the present invention provides an assay for TSH-R autoantibodies or TSH comprising:

(a) bringing into contact a test sample with cells from a clone expressing human TSH-R stably transfected with a reporter construct comprising cDNA of both a substrate capable of causing a measurable response when brought into contact with a corresponding protein and

a promoter containing cAMP response elements whereby increased cAMP levels increases expression of the enzyme;

(b) adding the corresponding substrate to cells thus contacted;

(c) measuring the response in the cells exposed to substrate; and

(d) comparing the response from test step (c) with the response from a standard or normal sample which has undergone steps (a) to (c).

Suitable reporter constructs are those in which the enzyme activity results in a colour change or emission of light. Examples of such enzymes include chloramphenicol acetyl transferase (CAT), Firefly luciferase, Renilla luciferase, ß-galactosidase, alkaline phosphatase, horse radish peroxidase or green fluorescent protein.

The cyclic AMP response element (CRE) of the reporter gene could be any promoter sequence or synthetic oligonucleotide which contains the CRE consensus sequence, TGACGTCA, preferably as a number of tandem repeats. A suitable promoter is that for the glycoprotein hormone alpha subunit which contains tandem cAMP response elements, described by Kay *et al* in Endocrinology 134 (2) 568-573 (1994). Another example is a construct driving the CAT enzyme which has been described by Chatterjee *et al* in Molecular Endocrinology 5 (1) 100-109 (1991).

However, the assay according to the present invention preferably comprises, in step (a), the use of a luciferase cDNA driven by a promoter containing cAMP response elements; and, in step (b), the use of luciferin;

which means that the response measured in steps (c) and (d) is light output from the luciferinised cells. Preferably, the luciferase is Firefly luciferase although Renilla luciferase or the like would also be suitable.

Most preferably, the reporter construct is α-luciferase, being a luciferase cDNA driven by the promoter for the glycoprotein hormone α subunit, mentioned above. For example, the plasmid pA3luc may be employed, having the glycoprotein hormone α subunit promoter introduced as described by Maxwell *et al* in Biotechniques 7 276-80 (1989). The α-luciferase is therefore 846 base pairs of 5' flanking sequence and 44 base pairs of exon 1 of the glycoprotein hormone α subunit promoter in the plasmid pA3luc. Alternatively, the CRE-containing sequence could be subcloned into a commercially-available luciferase reporter system such as the pGEM-luc vector from Promega. A further alternative is to use a plurality of plasmids such as in the system available from Stratagene (CREB reporting system, no. 219010), which includes plasmics enabling a luciferase response to be measured following an increase in cAMP.

The cells may be obtained as described in the reference mentioned above by Perret *et al*. Alternatively, the human TSH-R could be subcloned into any eukaryotic expression vector (of which pSVL is an example) available from Stratagene, InVitrogen or the like. For selectivity, the more recently-developed dual vectors which incorporate the antibiotic resistance gene within the same plasmid, such as pcDNAIII (available from InVitrogen) may be used. Otherwise, a separate plasmid for selection may be employed.

Preferably, the cells used in the assay (step (a)) are those identified as from clone JP09 in the above-mentioned reference which have been stably transfected within the order of 10^5 TSH-R per cell. More preferably, they are co-transfected with both α-luciferase cDNA and a puromycin resistance encoding plasmid such as pSV_2Neo (available from Clontech) to allow selection of assay cells with puromycin. Surviving cells are then tested for luciferase activity in response to TSH.

Preferably, the cells would be lyophilised (freeze-dried) and provided in individual containers with one container being used per assay.

Another co-transfection may be carried out to provide the assay with a method of correcting for the number of cells seeded in a well during use, in the case where non-lyophilised cells are to be used. Since the Renilla luciferase construct is constitutive and has different substrate requirements from Firefly luciferase, it provides such a method. The same value would be expected from every well whilst differences would reflect varying cell number. An appropriate plasmid for this transfection is the Renilla luciferase plasmid available from Promega, no. E2241. It contains the Herpes simplex virus thymidine kinase promoter upstream from Renilla luciferase, which is thus constitutively expressed.

Furthermore, when using intact rather than lyophilised cells, in order to prevent distortion of the assay by the presence of any TSH present in serum used in the cell culture medium, the serum should be charcoal-stripped at around 24 hours prior to assay.

The present invention therefore further provides a reporter construct comprising cDNA of both an enzyme capable of causing a measurable response when brought into contact with a corresponding protein and a promoter containing cAMP response elements whereby increased cAMP levels increases expression of the enzyme, in particular wherein the enzyme is a luciferase.

Accordingly, the present invention further provides cells from a clone expressing human TSH-R stably transfected with a reporter construct comprising cDNA of both an enzyme capable of causing a measurable response when brought into contact with a corresponding protein and a promoter containing CRE; the clone; and cDNA expressing the stably transfected human TSH-R.

Preferably, the assay according to this invention is carried out by means of a kit to enable fast and convenient results in a regular medicinal biochemistry laboratory or hospital pathology or diagnostic laboratory. The present invention therefore further provides a kit for carrying out the bioluminescent assay of the present invention, which kit comprises:

(a) cells from a clone expressing human TSH-R stably transfected with a reporter construct comprising cDNA of both an enzyme capable of causing a measurable response when brought into contact with a corresponding protein and a promoter containing cAMP response elements whereby increased cAMP levels increases expression of the enzyme;

(b) a standard sample for the assay;

(c) medium for culturing and/or reconstituting the cells; and

(d) instructions for carrying out the assay according to the present invention.

The corresponding protein and reagents relating thereto, and means for carrying out the response measurements may also be provided as part of the kit. For example, the kit may further comprise:

(e) buffer for lysing the cells;

(f) buffer for the reporter construct, preferably luciferase buffer; and

(g) corresponding protein, preferably luciferin, in buffer;

and, optionally, a luminometer.

Alternatively, for example, in the case where luciferase/luciferin are employed in the assay, a separate, commercially-available kit may be employed such as one of those available from the Promega Corporation. These commercially-available kits include no. E1483 wherein the luciferase is Firefly luciferase, and a dual-luciferase system no. E1910 which also employs Renilla luciferase.

The present invention will now be illustrated with reference to the following non-limiting examples:

Example 1: Preparation of cells for use in luminescent assay for TSH and TSH-R antibodies - Use of Firefly luciferase

Chinese hamster ovary cells (CHO-K1), available from the ATCC number CCL61, were subjected to calcium phosphate co-transfection, using standard protocols (Current Protocols in Molecular Biology, 1996, John Wiley & Sons Inc. section 9.1.4), with pSV_2Neo (available from Clontech) and a eukaryotic expression vector carrying the human TSH-R gene, pSVL-hTSHR (available from G. Vassart, IRIBIIN, Brussels, Belgium), as described by Perret *et al* (1990, *ibid*). The cells were selected with 400µg/ml geneticin (G418) and cloned by limiting dilution. Clone JP09 was isolated by this method (and is also available from Prof. G. Vassart), which expresses approximately 10^5 receptors per cell, as assessed in TSH binding experiments (described by Costagliola S, Swillens S, Niccoli P, Dumont J, Vassart G, Ludgate M in J Clin Endocrinol Metab 75 1540 *et seq.* (1992)).

Clone JP09 was co-transfected, again using the above-noted standard calcium phosphate method, with a eucaryotic expression vector carrying the puromycin resistance gene (available from, *inter alia*, InVitrogen, Stratagene etc.) and pA3Luc (available from V. Chatterjee, Univ. Cambridge, UK). Cells were selected in puromycin, 2.5µg/ml and cloned by limiting dilution. Clones were tested for light output (see Example 2) in response to bovine TSH (from Sigma, T8931). Clones giving a good TSH response were tested with a panel of normal human sera, the selection criterion in this case being a low light output of ≤ 1.5 relative light units.

Example 2: Preparation of cells for use in luminescent assay for TSH and TSH-R antibodies - Use of Firefly and Renilla luciferases

The method of Example 1 is followed, but after the second co-transfection involving the puromycin resistance gene, another co-transfection is carried out repeating the standard calcium phosphate method, but instead with a eukaryotic expression vector carrying the hygromycin resistance gene (available from Stratagene) and the R. luciferase plasmid E2241 (available from Promega).

Example 3: Assay for Measurement of (i) TSH bioactivity and (ii) Thyroid Stimulating (TSAB) or (iii) Blocking (TBAB) Antibodies

Culture medium for the cells is RPMI: 10% foetal calf serum (FCS); 1% glutamine; 1% pyruvate; 2% penicillin/streptomycin (and 2.5µg/ml puromycin, when amplifying cells for assay). 96 well plates are seeded with 5×10^4 cells prepared according to Example 1 and cultured overnight (approximately 16 hours) at 37°C in a water-saturated incubator. They are then cultured for a second night in medium containing 10% charcoal stripped calf serum (available from Gibco). Basal light output is measured, in triplicate wells, in the presence of 100µl of the medium containing 10% charcoal stripped serum. Standards (see below) and test samples are in the form of 10% patient serum to a final volume of 100µl of the medium containing 10% charcoal stripped calf serum. Basal, standard and test incubations are for 2-3 hours as above. The final result is expressed as relative light units (RLU) obtained by the ratio of standard:basal or test:basal.

(i) TSH bioactivity: this test is for patients having high circulating TSH levels when measured by radio-immunoassay but low circulating free T4, indicative of hypothyroidism because of defective TSH. Standards are well-characterised euthyroid and hypothyroid serum samples of increasing biological activity.

(iii) TSAB measurements are made in thyrotoxic patients. Standards are pooled normal human serum (having a light output of ≤1.5 RLU), bTSH and well-characterised TSAB containing sera of increasing activity.

(iii) TBAB measurements are made in the same patients as in (i), but the assay wells also contain 1mU/ml of bTSH. Standards are pooled normal sera + 1mU/ml bTSH and well-characterised TBAB containing sera of increasing activity.

Measurement of light output

Following the incubation period, supernatants are removed from the wells and the cells are washed twice in phosphate buffered saline. Light output is measured using a commercially available kit from the Promega Corporation, no. E1483, according to the manufacturer's instructions. This involves treating the cells in lysis buffer (Promega no. E1513), adding the Firefly luciferase reagent and measuring the light output in a luminometer.

If the cells used for the assay also express Renilla luciferase constitutively (as prepared in Example 2), to give a method of standardisation of cell number/well, the Promega Dual-Luciferase system is used, no. E1910.

In this case, following measurement of the Firefly luciferase as above, a reagent to quench the luminescent signal is added followed by the Renilla luciferase reagent and a second reading taken.